CRITICAL REVIEWS IN MICROBIOLOGY
2021, VOL. 47, NO. 2, 162–191
https://doi.org/10.1080/1040841X.2020.1863329

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REVIEW ARTICLE

Discerning the role of polymicrobial biofilms in the ascent, prevalence, and extent of heteroresistance in clinical practice

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ABSTRACT

Antimicrobial therapy is facing a worrisome and underappreciated challenge, the phenomenon of heteroresistance (HR). HR has been gradually documented in clinically relevant pathogens (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia* spp., *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Candida* spp.) towards several drugs and is believed to complicate the clinical picture of chronic infections. This type of infections are typically mediated by polymicrobial biofilms, wherein microorganisms inherently display a wide range of physiological states, distinct metabolic pathways, diverging refractory levels of stress responses, and a complex network of chemical signals exchange. This review aims to provide an overview on the relevance, prevalence, and implications of HR in clinical settings. Firstly, related terminologies (e.g. resistance, tolerance, persistence), sometimes misunderstood and overlapped, were clarified. Factors generating misleading HR definitions were also uncovered. Secondly, the recent HR incidences reported in clinically relevant pathogens towards different antimicrobials were annotated. The potential mechanisms underlying such occurrences were further elucidated. Finally, the link between HR and biofilms was discussed. The focus was to recognize the presence of heterogeneous levels of resistance within most biofilms, as well as the relevance of polymicrobial biofilms in chronic infectious diseases and their role in resistance spreading. These topics were subject of a critical appraisal, gaining insights into the ascending clinical implications of HR in antimicrobial resistance spreading, which could ultimately help designing effective therapeutic options.

ARTICLE HISTORY
Received 3 June 2020
Revised 3 December 2020
Accepted 6 December 2020
Published online 27 January 2021

KEYWORDS
Antimicrobial; biofilm; clinical failure; heteroresistance; interspecies interactions

Antimicrobial resistance

Since the discovery of penicillin in the 1920s (Fleming 1929), we have become aware of how well microorganisms find ways to circumvent every treatment thrown their way. Antibiotics saved countless lives and took us to a "golden era" of medicine, but the emergence of antimicrobial resistance (AMR) quickly led us to a time where previously treatable infections are becoming life-threatening once again (Martens and Demain 2017; CDC 2019). AMR has been increasingly impacting human, animal, and environmental settings (Aslam et al. 2018), with estimates pointing to 7 M yearly deaths from infections caused by resistant pathogens (IACG 2019).

Behavioural and social-economic factors, including antibiotic over consumption, lack of precise drug usage, poor sanitation and hygiene practices, access to counterfeit drugs, and increased international transportation, have helped AMR to spread (Laxminarayan and Heymann 2012; Ferri et al. 2017; CDC 2019; EFSA 2019). In healthcare settings, the burden of AMR is substantial, with high antibiotic intake, patient vulnerability, common use of invasive practices, and enduring influx of pathogens being the root causes (Golkar et al. 2014; Roca et al. 2015). The emergence of multiple resistance mechanisms has seriously thwarted efforts to achieve therapeutic success. Clearly, resistant microorganisms have been and will continue to be a common cause of treatment failure. The challenge is greatest for critically ill and immunocompromised patients, though the rising AMR puts everyone at risk. If no urgent actions are taken, AMR will cause an estimated 10 M deaths by 2050 (IACG 2016).

Clarifying terminologies: resistance, heteroresistance, tolerance, and persistence

The complexity behind the mechanisms by which microorganisms evade antimicrobials has led to the creation of several terminologies and definitions that multiplied and evolved as new information was outputted.

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Given the ever-growing intricacy of this subject, definitions are often overlapping, making it hard to distinguish certain concepts and causing unfortunate misinterpretations. It is crucial that these definitions are clear, so researchers can rely on what is published to advance antimicrobial research. Table 1 summarizes key differential aspects of the concepts of resistance, heteroresistance (HR), tolerance, and persistence, which are further explored next.

Resistance

Resistance can be classified as intrinsic, acquired, or adaptive, being usually present concurrently in more than one of these formats in a given microorganism. Intrinsic resistance is the innate ability of a microorganism to resist an antimicrobial because of inherent structural or functional traits coded in their genome (Blair et al. 2015). Examples include cell-wall composition (e.g. Gram-negative *vs* Gram-positive bacteria), outer membrane permeability (e.g. restrictive protein channels), drug efflux pumps (e.g. MexAB-OprM system in *Pseudomonas aeruginosa*), and expression of intrinsic antibiotic resistance genes (e.g. β-lactamases) (Jorge et al. 2019).

In turn, acquired resistance illustrates the transformation of an originally sensitive microorganism into a resistant one. This can arise through the acquisition and incorporation of new genes (carried in plasmids, transposons, integrons, or prophages) from neighbouring microorganisms by horizontal gene transfer (e.g. spread of β-lactam resistance among different species). However, it can also be a result of genetic mutations (e.g. loss of OprD porin via mutation in the *oprD* gene in *P. aeruginosa*) (Taylor et al. 2014; Pang et al. 2019). Both intrinsic and acquired resistances are stable and can be transmitted vertically (Blair et al. 2015).

Adaptive resistance is a more complex form of resistance. Here, microorganisms alter their gene/protein expression in response to an antimicrobial or environmental factor (e.g. pH, temperature, nutrient or oxygen concentration) (Jorge et al. 2019). Contrary to intrinsic and acquired resistances, adaptive resistance is typically described as unstable, transient, and dependent on the presence of the triggering factor. The high survival rate and its temporary nature indicates a non-genetic causation, making it not vertically transmittable (Sandoval-Motta and Aldana 2016). Microorganisms revert to their susceptible state (although sometimes not entirely) after antibiotic exposure is ceased (Arzanlou et al. 2017; Pang et al. 2019). This type of resistance is intricate and not restricted to its more common description. In fact, adaptive resistance is mechanistically diversified and sometimes overlaps with other types of resistance. Mechanisms of adaptive resistance can include epigenetic inheritance, heterogeneity of gene expression, high mutability, population heterogeneity, gene amplification, efflux pumps, and biofilm formation (Jorge et al. 2019). For example, some antibiotics can induce a hypermutator state due to the activation of the SOS response. Most of these mutations are not stable due to fitness costs, even though some may confer

Table 1. Overview of resistance, HR, tolerance, persistence definitions.

Concept	Characteristics	Transmitted vertically?	Growth?	Detection methods
Resistance				
Intrinsic	• Encoded in genome • Innate properties	Yes	Yes	MIC
Acquired	• Resistance genes acquired from other microorganisms (horizontal transfer) or stable mutations			
Adaptive	• Dependent on triggering factor • Temporary (mostly) until triggering factor is cut-off	No (mostly)		
HR	• Subpopulation(s) • Polyclonal HR: mixed populations or rare resistant mutants (acquired resistance) • Monoclonal HR: pure clones; can be stable or unstable	Yes (if stable)	Yes	MIC or MBC Disc diffusion E-test PAP
Tolerance	• Survival to transient exposure • Time-dependent (takes longer for antibiotic to kill cells) • Same MIC as susceptible cells • Tolerance by slow-growth: inherited non-inherited Tolerance by lag	Yes (if inherited)	Yes	Time-kill curve MDK
Persistence	• Subpopulation • Metabolically inactive cells • Same MIC as susceptible cells • Time-dependent persistence: subpopulation of tolerant bacteria • Dose-dependent persistence: transient decrease in antibiotic sensitivity	No	No	Time-kill curve

HR: Heteroresistance; MBC: minimum bactericidal concentration; MDK: minimum duration for killing; MIC: minimum inhibitory concentration; PAP: population analysis profile.

resistance. However, if the antibiotic stimulus endures, there is an increased probability of a stable mutation that confers resistance to occur. This is an example of how the concepts of acquired and adaptive resistance may overlap. Adaptive resistance can also be a bridge between acquired and intrinsic resistance, since genetic mutations or epigenetic changes caused by environmental signals can alter the expression of intrinsic resistance mechanisms (Sandoval-Motta and Aldana 2016). Another example of overlapping concepts is given by the important role of porins and efflux systems. These typical traits of intrinsic resistance play a crucial role in adaptive resistance, since antibiotics can induce the latter by causing an overexpression of those traits (Fernandez and Hancock 2012).

Heteroresistance

HR is broadly defined by the presence of one or more microbial subpopulations with higher levels of resistance then the remaining population. In other words, it describes heterogeneous microbial populations, wherein a subset of cells exhibits lower susceptibility to an antimicrobial (Saravolatz et al. 2014). Several factors need to be considered when characterising this phenomenon. HR may be polyclonal or monoclonal. Polyclonal HR is related to heterogeneous consortia with genetically distinct subpopulations or to the appearance of rare resistant mutants whose frequency increases with antibiotic exposure. In turn, HR is monoclonal if present in pure clones (Andersson et al. 2019). Most published studies report or study monoclonal HR caused by genetic or phenotypic heterogeneity (Dewachter et al. 2019).

Depending on the mechanism behind it, HR can be stable, i.e. the resistance of the subpopulation does not revert after antibiotic removal, or unstable, i.e. the subpopulation resistance decreases or reverts to susceptible upon antibiotic removal. The level of resistance (explored further below) and the frequency of the resistant subpopulation are two other factors to consider when characterising HR (Andersson et al. 2019). Noteworthy, the frequency of HR relies upon several variables, including clinical strains involved, antimicrobial agent, detection method, and the criterion used to define it (Nicoloff et al. 2019). To better understand the factors used to fully characterize HR, the review from Dewachter et al. (2019) is recommended.

Tolerance

Tolerance is the capacity of a microbial population to survive a transient exposure to an antimicrobial, even in concentrations above the minimum inhibitory concentration (MIC). Unlike resistance, tolerance is temporary, since it just takes more time to kill the cells. There are two types of tolerance, namely tolerance due to slow growth and tolerance due to lag. Tolerance by slow growth can be further divided into inherent, i.e. characteristic of a given species/strain (e.g. long duplication time), and non-inherent, i.e. caused by poor growth conditions (e.g. biofilms), stress factors (e.g. antibiotics), or microbial stationary growth phase. In turn, tolerance by lag occurs when an antimicrobial is applied to a microbial population in their lag growth phase (Brauner et al. 2016; Balaban et al. 2019; Jorge et al. 2019). Since tolerant and susceptible cells can exhibit the same MIC, the recommended method to identify tolerance is time-kill monitoring with assessment of the minimum duration for killing (MDK). It is also important to refer the concept of dormancy, which can be viewed as an extreme case of tolerance by slow growth, i.e. growth rate equal to zero (Brauner et al. 2016).

Persistence

Persistence, similarly to HR, is characterized by population heterogeneity. A major differential aspect between both phenomena is that while heteroresistant cells grow during antimicrobial treatment, persisters do not, given their inactive metabolism (Dewachter et al. 2019; Jorge et al. 2019). The pattern of antimicrobial sensitivity in persisters remains identical to that of the main population (Gefen and Balaban 2009). Persisters consist of a phenotypic unstable variant, comprising about 1% of the total population. Persistence can be time- or dose-dependent. Time-dependent persisters are tolerant cells, so everything abovementioned about tolerance applies. In turn, dose-dependent persisters have a transiently overexpression of a resistant factor that makes them less susceptible, being necessary to apply higher antimicrobial concentrations to kill them (Brauner et al. 2016; Dewachter et al. 2019).

Prime factors engendering HR divergent interpretations

As stated, HR denotes, in its broadest sense, population-wide variable responses towards a particular antimicrobial (El-Halfawy et al. 2013). A formally clear and consistent accepted definition for HR remains unknown, owing to contradictable, variable, and confounder factors, including: i) non-standard criteria (e.g. lack of drug concentration ranges or cut-off concentrations); ii)

absence of standardized and accurate diagnostic methodologies; and iii) presence of other forms of heterogeneity.

The absence of concentration ranges in HR definition renders it sketchy and may exclude cases of subpopulations with various resistance levels integrated in a sensitive or resistant population (Li et al. 2006; Falagas et al. 2008). In other words, the original culture can be: i) sensitive to an antimicrobial, harbouring a less sensitive subset of cells that respond to concentrations below the breakpoint; ii) resistant, but comprising a minor subpopulation with higher resistance level; or iii) majorly sensitive, incorporating a small fraction of cells that are drug resistant (El-Halfawy and Valvano 2015). The latter case is the classic and most commonly reported form of HR. It is less likely to be detected by traditional susceptibility testing, posing serious threats to antimicrobial treatment due to the selection of the resistant subpopulations by inappropriate drug dosage (Li et al. 2017). Thus, specifying the concentration intervals for which a subpopulation is considered resistant would allow to recognize the various HR levels and identify the form of HR present (Li et al. 2006; Halaby et al. 2016).

Oftentimes, rather than include the actual quantified resistance levels of the population and subpopulation, HR is only described as fold increases in the MIC of the main population. Here, the single clinical breakpoints, obtained from traditional *in vitro* susceptibility testing defined by official bodies (e.g. CLSI, CLSI 2016; EUCAST, Matuschek et al. 2014), are used as cut-off values to distinguish between homogeneous and heterogeneous populations. Note that, depending on the method, cut-off concentrations (Sader et al. 2009; Hermes et al. 2013) or cut-off diameters can be adopted (Khan et al. 2008). To avoid inconsistent HR readings, a definition was proposed stating that the presence of a "subpopulation of cells capable of growing under concentrations of the antimicrobial drug, at least, eightfold higher than the highest non-inhibitory concentration of the dominant population" indicates HR (El-Halfawy et al. 2013). Other researchers opt to consider all the microbial colonies falling into zone diameters of 10 mm in disc diffusion assays as heteroresistant (Hallander and Laurell 1972).

Another factor leading to changeable HR definitions is the lack of standardized protocols and/or detection limits of the methodologies used to identify HR (El-Halfawy and Valvano 2015). The current "gold" standard test is the population analysis profile (PAP), consisting of estimating cell counts growing in increasing concentrations of the antimicrobial (Søgaard 1985; Søgaard and Gahrn-Hansen 1986; El-Halfawy et al. 2013). Although reliable and reproducible, this method is labour-intensive, time-consuming (3–5 days), and costly, being cumbersome to implement (Satola et al. 2011; Van Hal et al. 2011). PAP is usually accomplished with twofold increments in antimicrobial concentration to evaluate bacterial growth on agar plates. Nevertheless, HR frequency, i.e. fraction of the resistant subpopulation growing under antibiotic pressure, varies with the antimicrobial dose used, precluding comparison between studies measuring frequencies at different drug intervals. To make comparisons possible, Andersson et al. (2019) suggests reporting these frequencies at concentrations eightfold above the resistance level of the main population.

In vitro susceptibility tests routinely used to identify HR include broth microdilutions, disc diffusions, and E-tests (Andersson et al. 2019). These methods are faster and less expensive than PAP, but entail some pitfalls. In broth microdilution assays, the "skip wells" phenomenon (isolated wells exhibiting growth in higher concentrations) (Landman et al. 2013; Guérin et al. 2016) contributes to uninterpretable and irreproducible MIC results. In turn, agar methods, such as disc diffusion and E-tests, have limited sensitivity. HR goes mostly unnoticed in routine antibiograms, as the fraction of the resistant subpopulation is extremely low (Lo-Ten-Foe et al. 2007). To allow time for proliferation of less abundant but more resistant population members, some researchers suggested prolonging the incubation time (48 h) and inspecting HR at later time points (Li et al. 2006; Pournaras et al. 2010; Band et al. 2018). However, care should be taken when microtiter plates are incubated for long periods. Long incubations increase the risk of contamination of the susceptible population and the likelihood of *de novo* mutants to arise, resulting in an inaccurate HR identification. Additional methods (automated systems and molecular detection assays) with potential to diagnose HR have evolved in the last years (e.g. VITEK 2, MicroScan, WalkAway, GenoType MTBDRplus) (Andersson et al. 2019). Despite, this does not displace the demand of a practical (i.e. accurate, rapid, sensitive, and cost-effective) and reliable detection method for routine use in clinical settings.

Finally, the presence of other forms of heterogeneous behaviour in microbial populations may instigate confusion, generating false HR positives. This can be discerned, for instance, when a microbial population exhibits bimodal growth in PAP curves (Kondo et al. 2001). This "Eagle effect" (Eagle and Musselman 1948; Jarrad et al. 2018) describes a paradoxical "more-kills-

less" response, wherein a microorganism displays improved survival when exposed to supra-minimum bactericidal concentration (MBC) concentrations. Extensive reports have showed this effect in a wide range of microorganisms (Lorian et al. 1979; Fleischhacker et al. 2008; McKay et al. 2009; Wu et al. 2015), though the mechanism behind it remains unclear.

HR reported in clinically significant pathogens

Healthcare-associated infections (HAIs) remain a major worldwide concern, contributing to prolonged hospital stay, patient disability, and economic burden (Khan et al. 2015, 2017). Several microorganisms, including *P. aeruginosa*, *Staphylococcus aureus*, *Burkholderia* spp., *Acinetobacter baumannii*, and *K. pneumoniae*, possess the ability to endure in medical devices (e.g. urinary catheters, endotracheal tubes, feeding tubes, vascular lines) (Safdar and Maki 2002). Because of their opportunism, these bacteria have been implicated in severe nosocomial outbreaks (e.g. bloodstream infections, pneumonia, urinary tract infections, endocarditis). They can trigger chronic or recurrent life-threatening infections in critically ill and immunosuppressed patients, causing high mortality and morbidity rates (Davies and Davies 2010). The selective pressure provided by the continuous antibiotic exposure is a key risk factor for AMR and even for multidrug resistance (MDR) development. Worryingly, the majority of the aforementioned bacteria are priority targets, according to the World Health Organisation (WHO), stressing the need for new antibiotics effective against multidrug and extensively drug resistant pathogens (WHO 2017).

Despite underappreciated, fungal infections also have unequivocally clinical significance and substantial burden in healthcare systems. The widespread antibiotic misuse coupled with indwelling devices provides an optimal environment for opportunistic co-colonization and proliferation of fungal species (Ganguly and Mitchell 2011; Weiner et al. 2016; Kean et al. 2018). Amongst fungi regarded as human pathogens, the members of the genus *Candida* are the most frequent. *Candida albicans* has long been the prevailing species co-isolated from polymicrobial infections, interacting with bacteria and playing key roles in bacterial-fungal illnesses (Nseir et al. 2007; McCormack et al. 2015; Townsend et al. 2016; Lopes, Rodrigues, et al. 2017). In the last decades, however, the emergence of infections due to other *Candida* species, such as *C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei*, has increased significantly (Manzano-Gayosso et al. 2008; Negri et al. 2012; Silva et al. 2012).

Despite HR being first reported more than 50 years ago (Alexander and Leidy 1947; Sutherland and Rolinson 1964; Kayser et al. 1970), the truth is that HR prevalence has been disregarded from clinical studies, likely due to its poor understanding. However, we have witnessed researchers taking growing interest on this topic, especially over the last two decades (Figure 1). HR is becoming a widely accepted problem in healthcare settings, with incidences arising worldwide. Following up on a preceding report reviewing HR incidences in important bacterial pathogens (El-Halfawy and Valvano 2015), we hereby gathered the most recent HR occurrences from the last 5 years (2015–2020). Table 2 summarizes HR incidences in clinically relevant pathogens, including bacteria and fungi, to varied antimicrobial categories. Excluding *Burkholderia* spp., for which no new HR cases were found, HR episodes have hugely increased worldwide, likely resulting from the increasing conscientiousness regarding the clinical meaningful of this phenomenon. A summary of the findings is described next.

Acinetobacter baumannii

Carbapenems are the first choice to treat MDR *A. baumannii* infections, but resistance towards them has been emerging worldwide (Higgins et al. 2010). Concerning HR to carbapenems in *A. baumannii*, one study reported the selection of imipenem-heteroresistant subpopulations supposedly induced by previous antibiotic pressure (Li et al. 2017). A complete genome sequencing analysis identified a small number of insertion sequences in the heteroresistant strain as well as many mobile genetic elements, however more closely associated to standard resistant than to heteroresistant phenotypes. Hence, the HR mechanisms were not entirely clear in this study.

There has also been an alarming emergence of HR in *A. baumannii* to colistin, a last-resort drug. Regrettably, the underlying mechanisms are also not yet fully understood. Although not fully elucidated the primary mode of action of colistin, the lipid A in the LPS of the outer membrane has been recognized as its main target in Gram-negative bacteria (Formosa et al. 2015). Colistin acts by displacing the lipopolysaccharide (LPS) content and solubilising the outer bacterial membrane (Murray and Hospenthal 2005). The active role of both PmrAB and PhoPQ systems in regulating colistin HR has been disclosed, thus leading to membrane/cell wall driven phenotypes. In the case of *A. baumannii*, mutations in

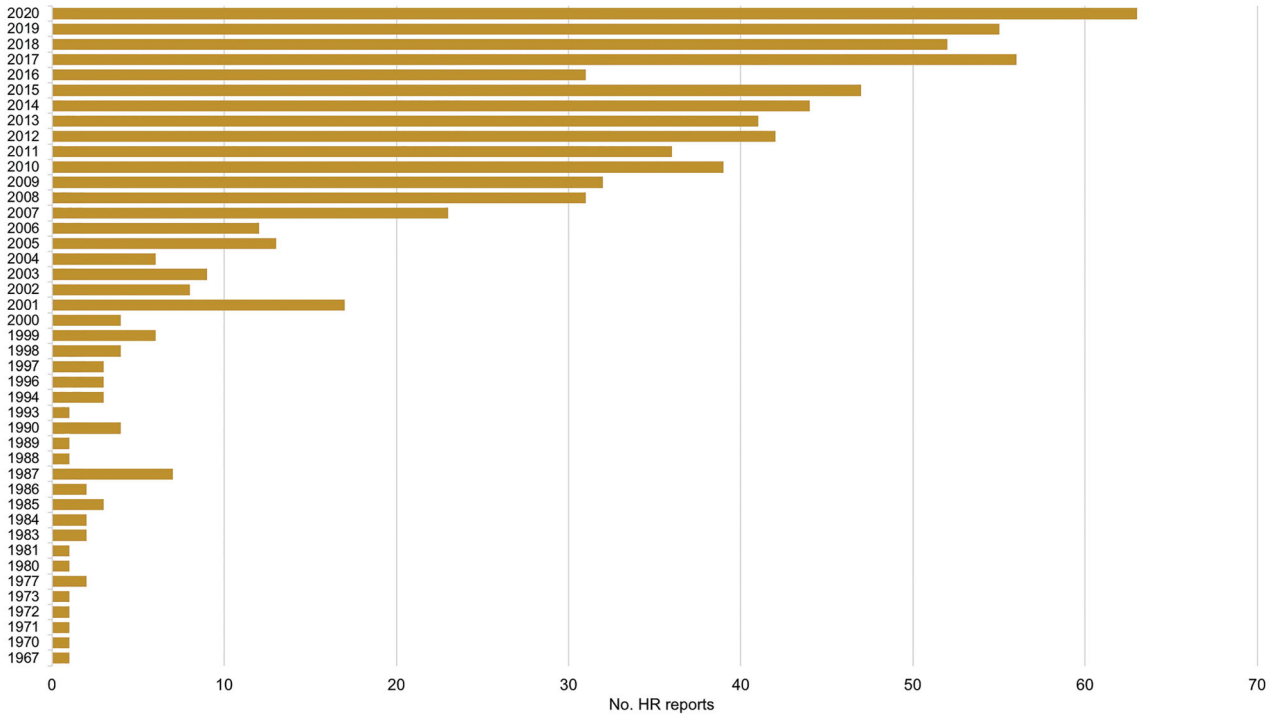

Figure 1. Overview of the number of HR reports over the years. Total number of reports listed in PubMed, embracing the keyword "Heteroresistance" (last data accessed: October 12 2020).

genes linked to the PmrAB regulatory system (*pmrA/B lpxA/D/C*), implicated in modifying lipid A biosynthesis and downregulating the LPS core lipid A regions (Zhang et al. 2017; Charretier et al. 2018; Rodriguez et al. 2019; Chen et al. 2020), have been detected in heteroresistant isolates along with upregulated expression of efflux pump genes (Machado et al. 2018; Chen et al. 2020). This denotes that *A. baumannii* may exhibit both homogeneous resistant and HR phenotypes, while presenting mutations in the same targets (Machado et al. 2018). Similarly to what occurs under carbapenems therapy, colistin pressure may lead *A. baumannii* populations to harbour a small subpopulation of highly colistin-resistant variants (Li et al. 2006; Moffatt et al. 2010).

Despite the well-known aminoglycoside resistance phenotype of *A. baumannii*, aminoglycoside HR is rare in this species. Interestingly, a recent report concerning tobramycin HR in *A. baumannii* strain AB5075 (Anderson et al. 2018) uncovered distinct two-way underlying mechanisms (shown to be highly unstable), revealing a far more complex process than previously thought. The first mechanism was acquired through amplification of the *aadB* gene, a 2″-nucleotidyltransferase carried in the large plasmid p1AB5075, conferring resistance to tobramycin and to gentamicin. Although uncertain, the second was a RecA-independent mechanism, unlinked from amplifications of the *aadB* region.

Klebsiella pneumoniae

Regarded as an emerging cause of severe HAIs (e.g. urinary tract, bloodstream, soft tissues) (Podschun and Ullmann 1998), carbapenem-resistant *K. pneumoniae* (CRKP) remains in the top urgent threats listed by the WHO (2017). The analysis of several meropenem-heteroresistant mutants showed that the major mechanism is a deficiency in porin gene (*ompK*36) expression, followed by IS1 insertions and no detectable OmpK36 protein. The high mutation frequencies (10^{-5} to10^{-4}) are worrisome and sound the alarm on the risk of developing carbapenem resistance during therapy (López-Camacho et al. 2019).

Similarly to *A. baumannii*, a rapid dissemination of colistin HR has been increasingly reported. Diverse studies relate multiple overlapping mechanisms driving for both colistin-heteroresistant and resistant phenotypes in *K. pneumoniae*, generally engendered *via* mutations in the PhoPQ and PmrAB two-component systems and/or in the regulator gene *mgrB* (Berglund 2019). A likely mechanism can be advocated similarly to that described for many colistin-heteroresistant Enterobacteriaceae strains (Kang et al. 2019). Their decreased susceptibility to polymyxins, namely to colistin, has been mainly attributed to the chemical modification of the lipid A. This occurs through the biosynthesis of abnormal cationic amine moieties of 4-amino-4-deoxy-L-arabinose (l-Ara4N) and/or

Table 2. Cases of HR reported in clinically significant pathogens between 2015 and 2020.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
A. baumannii	Aminoglycosides	Tobramycin	Strain AB5075	E-test PAP	Genotypic	• Amplification of the *aadB* resistance gene • Unknown RecA-independent mechanism • Unstable HR	Anderson et al. (2018)
	Antimicrobial peptides	Colistin	Clinical isolates (blood) (Korea)	BMD Agar screen PAP	Genotypic	• Two distinct HR patterns observed: one confirmed by PAP (Type I) and the other confirmed by screening in COL plates (Type II) • Type I isolates showed resistance upon exposure to high colistin concentrations (stable HR) • Type II isolates not identified at high colistin concentrations (unstable HR) • Amino acid changes in PmrAB and LpxACD were observed for both HR patterns	Hong et al. (2020)
			Carbapenem-resistant clinical isolates (varied sources) (Thailand)	BMD PAP	Unknown	• 33 isolates (44%) exhibited HR	Thet et al. (2020)
			Clinical isolates (China)	BMD PAP	Genotypic	• Mutations in *lpxACD* genes • Overexpression of efflux pump genes • Heteroresistant mutants showed MDR and regrow upon 12h colistin stress	Chen et al. (2020)
			Carbapenem-resistant isolates (varied sources) (Iran)	BMD PAP	Unknown	• Nine in 44 isolates showed HR (subpopulations grown under 6–8 μg/mL colistin)	Ezadi et al. (2020)
			Clinical isolates (varied sources) (Turkey)	BMD E-test PAP	Unknown	• HR observed for 21.4% of the isolates	Çağlan et al. (2019)
			Clinical isolates (varied sources) (Argentina)	Broth dilution PAP	Genotypic	• Fifteen isolates showed HR • Low HR frequency ($1x10^{-4}$ to $1x10^{-5}$) • Mutations in *lpxA*, *lpxC* and *in* pmrB were detected • Certain isolates showed slow growth	Rodriguez et al. (2019)
			MDR clinical isolate (bronchial secretion)	E-test PAP	Genotypic	• Overexpression of efflux pump genes: *adeB, adeJ, adeG, craA, amvA, abeS and abeM* • Stable HR	Machado et al. (2018)
			MDR clinical isolate (Switzerland)	BMD E-test PAP	Genotypic	• Mutations outside the histidine kinase domain of the pmrB gene in the PmrAB regulatory system • Overexpression of the PmrCAB operon • Unstable HR (one mutant)	Charretier et al. (2018)
			Clinical isolates (varied sources) (USA)	Agar screen E-test	Unknown	• HR exhibited in 83% of the isolates	Srinivas et al. (2018)
			Clinical isolates (blood, skin swabs and tracheal aspirates)	BMD E-test PAP	Unknown	• Almost all isolates showed HR (4 were persisters) • Polyclonal HR	Choi et al. (2017)
		Polymyxin B	Clinical isolates (Brasil)	PAP Agar screen	Unknown	• Polymyxin B HR found in 13/21 (63%) of the isolates • PAP HR frequency ranged from 10^{-7} to 10^{-4}	Genteluci et al. (2020)

(*continued*)

Table 2. Continued.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
	B-lactams	Imipenem	Clinical isolate (sputum sample) (China)	Disc diffusion PAP	Genotypic	• Presence of IS elements: ISAba1, ISAba22, ISAba24 and ISAba26 • 19 genes related to resistance to 8 antimicrobials • 11 genomic islands, some containing ISs and resistance determinants	Li et al. (2017)
	Varied classes	Amikacin Cefepime Gentamicin Imipenem Netilmicin SMX Tobramycin	Clinical isolates (varied sources)	E-test PAP	Genotypic	• Sulfamethoxazole and amikacin HR were unstable and linked to gene amplifications or gene mutations	Nicoloff et al. (2019)
Burkholderia spp.	Not annotated						
Candida spp.	Antifungals	Fluconazole	*C. glabrata* clinical isolates (UK)	BMD PAP	Genotypic and epigenetic	• Upegulation of CDR1 and PDH1 genes, encoding ATP-binding cassette transmembrane transporters • Phenotypic clustering and variations in HR within clonal groups, suggest both genetic and epigenetic HR determinants • HR was associated with persistent viable cells in mouse kidney tissue during treatment • Stable HR	Ben-Ami et al. (2016)
K. pneumoniae	Antimicrobial peptides	Colistin	ESBL-producing clinical isolates (Chile)	BMD PAP	Genotypic	• Stable HR phenotype was found for 8/60 strains • HR frequencies varied among 10^{-5} to 10^{-7} • All colistin-HR exhibited MDR phenotype and were resistant to several antibiotics • Mutations in the two-component regulatory systems PmrAB and PhoPQ, as well as a disruption of the mgrB global regulator mediated by IS1-like and IS-5-like elements	Morales-León et al. (2020)
			Clinical isolates (blood) (Korea)	Disc diffusion PAP	Genotypic	• Seven amino acid substitutions found in PmrAB and PhoPQ regulatory systems	Cheong et al. (2019)
			Two clinical isolates (Mu9 and Um 156)	E-test PAP	Genotypic	• Involvement of the PhoPQ signalling, though repression of the mgrB and upregulation of the *phoP* genes • HR associated to *in vivo* treatment failure (mouse model) • Unstable HR	Band et al. (2018)
			Clinical isolates (blood cultures) (Hungary)	Agar screen	Unknown	• Heteroresistant subpopulations found in 17% of the isolates • Low HR frequencies (1.25×10^{-8} to 1.1×10^{-5}) • Transposition of *mcr-1* to the chromosome as a possible HR mechanism	Juhász et al. (2017)

(*continued*)

Table 2. Continued.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
			Clinical isolate (France)	E-test	Genotypic	• Single nucleotide insertion in mgrB, leading to a premature stop codon and an inactive MgrB regulator	Bardet et al. (2017)
			ESBL-producing clinical isolate (MDR) (South Africa)	BMD PAP	Genotypic	• Presence of 5 HR subpopulations (MICs: 8 to 64 mg/L) • SNPs identified in certain isolates • Mutations in the lpxM, *mgrB, phoQ,* and *yciM* genes	Halaby et al. (2016)
			Clinical isolate (South Africa)	E-test BMD	Genotypic	• Single amino acid substitution (Asp191Tyr) in the PhoP protein • Unstable HR	Jayol et al. (2015)
	B-lactams	Ertapenem Imipenem Meropenem	ESBL-producing clinical isolates	Disc diffusion BMD PAP	Unknown	• A total of 55 ESBL strains (in which, 6 were KP) showed HR to at least one carbapenem	Tan et al. (2020)
		Meropenem	OXA-48-producing clinical isolates	PAP	Genotypic	• Deficiency in *ompK36* porin gene conferred HR	López-Camacho et al. (2019)
	Tetracyclines	Eravacycline	Clinical isolates (varied sources) (China)	Agar screen PAP	Genotypic	• Overexpression of OqxAB and of its transcriptional regulator RamA, as well as of MacAB efflux pumps	Zheng et al. (2018)
	Varied classes	Amikacin Aztreonam Cefepime Ertapenem Gentamicin Netilmicin SXT Tetracycline Tobramycin	Clinical isolates (varied sources)	E-test PAP	Genotypic	• Unstable HR associated with resistance gene amplifications deteced for gentamycin, tobramycin, netilmicin and sulfamethoxazole (SXT) • Amikacin HR unstable, governed by gene mutations	Nicoloff et al. (2019)
	Antimicrobial peptides	Colistin	Clinical isolates (China)	BMD PAP	Genotypic	• Major PmrB substitutions detected in 8 of 9 isolates • Two isolates had PhoQ alterations • Alterations in two novel systems (ParRS and CprRS) • Additional 4-amino-4-deoxy-L-arabinose (l-Ara4N) moieties shown in lipid A profiles of heteroresistant isolates	Lin et al. (2019)
P. aeruginosa			Clinical isolates (blood cultures) (Hungary)	Agar screen	Unknown	• Low HR frequency (1.8×10^{-7} to 3×10^{-4})	Juhász et al. (2017)
	β − lactams	Imipenem	Clinical isolates (China)	Disc diffusion PAP	Genotypic	• Down-regulation of *oprD* contributed to imipenem resistance and HR • Up-regulation of efflux pump coding genes (*mexE* and *meY*)	Xu et al. (2020)
		Cefepime	Clinical isolates (bacteraemia) (China)	Disc diffusion E-test PAP	Genotypic	• *AmpC* cephalosporinase hyperproduction may have contributed to HR • Upregulation of *mexX* efflux gene (three isolates) • Stable HR	Jia et al. (2020)
		Imipenem Meropenem	Clinical isolates (China)	Disc diffusion PAP	Genotypic	• Low HR frequency • Overexpression of efflux genes: *mexB, mexC, mexE, and* mexX • Decreased OprD porin	He et al. (2018)

(*continued*)

Table 2. Continued.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
		Imipenem	Clinical isolates (China)	Disc diffusion PAP	Genotypic	• Down-regulation of oprD contributed to imipenem resistance and HR • Up-regulation of efflux pump coding genes (mexE and meY)	Xu et al. (2020)
			Clinical isolates (China)	Disc diffusion	Genotypic	• Upregulation of MexAB efflux pump in heteroresistant isolates	Mei et al. (2015)
	Several classes	Aztreonam Cefepime Ceftazidime Ciprofloxacin Imipenem Tobramycin TZP TMP-SMX	Clinical isolates (CF) (USA)	E-test	Genotypic	• Mutations in *gyrA and* gyrB contributed to HR • Truncation of OprD porin likely related to carbapenems HR • Overexpression of efflux MexAB-OprX correlated with TMP-SMX HR	Qin et al. (2018)
	Phosphonic acid derivative	Fosfomycin	MDR and non-MDR clinical isolates (critically ill patients, including CF) (Australia)	Agar screen PAP	Unknown	• All isolates exhibited HR • Low HR frequencies (1.10×10^{-6} to 3.69×10^{-4}) • HR was the probable cause of regrowth in the post-antibiotic effect	Walsh et al. (2015)
S. aureus	Glycopeptides	VAN	MRSA isolates (bacteraemia and resolved bacteraemia) (South Korea)	E-test PAP	Genotypic	• hVISA was detected in 28.1%, 23.4% and 21.3% of long-term, persistent and resolved MRSA bacteraemia • The *SCCmec* II and agr group II genotypes more frequent in the long-term bacteraemia group	(Lee et al. 2020)
			Clinical isolates (USA)	E-test PAP	Phenotypic and Genotypic	• Mutations detected in the two-component histidine kinase sensor gene • Detection of *vraS* and *in* rpsU genes • Thicker cell wall and slower growth confirmed in heteroresistant isolates • Many genes related to amino acid metabolism and cell fitness downregulated	Basco et al. (2019)
			Clinical isolates (bloodtream infections) (Brasil)	Agar screen PAP	Unknown	• Six isolates, among 200, exhibited HR	Damasco et al. (2019)
			MRSA isolates (bloodstream infection)	PAP	Genotypic	• Mutations in *vraSR*, *graSR*, *walKR*, and/or tcaRAB genes • Mutation in a teicoplanin *resistance-associated* (tcaRAB) operon	Bakthavatchalam et al. (2018)
			MRSA isolates (endocarditis and pneumonia) (USA)	PAP	Unknown	• About 19% were hVISA isolates	Trinh et al. (2018)
			MRSA isolate (Spain)	E-test	Unknown	• hVISA was *agr* I and SCCmec type IV positive	Varona-Barquín et al. (2017)
			Clinical isolates (bacteraemia) (Korea)	BMD E-test PAP	Genotypic	• HR detected in 49% of the isolates • Development of HR during persistent bacteraemia occurred in 4 (16%) among 25 isolates, and acquisition *of* agr dysfunction occurred in 2 (16%) among 12 *initial* agr-functional isolates	Kim et al. (2017)

(*continued*)

Table 2. Continued.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
			MRSA isolates (bloodstream infection) (Albany)	PAP	Unknown	• hVISA emerged in 7 of 119 (5.9%) patients • HR emergence partially explained by suboptimal exposure to vancomycin at initial days of therapy	Martirosov et al. (2017)
			Clinical isolates (leukaemia) (Argentina)	BMD PAP	Genotypic	• Continuous exposure of isolates to antibiotic treatment could enhance IS256 transposition, being responsible for the eventual loss of *agr* function	Di Gregorio et al. (2016)
			Clinical isolates (pneumonia)	BMD E-test	Unknown	• Isolates with low cytotoxicity exhibited a greater prevalence of vancomycin HR, being recovered more often from older and frailer patients	Rose et al. (2015)
			MRSA isolates (bacteraemia)	E-test PAP	Unknown	• Prior exposure to vancomycin at low concentrations likely facilitated HR emergence	Khatib et al. (2015)
			Clinical isolates (pneumonia)	PAP BMD	Unknown	• Mortality rates and vancomycin treatment failure in patients were suspectidly associated to hVISA	Claeys et al. (2016)
			MRSA iolates (varied sources) (Taiwan)	E-test PAP	Genotypic	• Growing hVISA prevalence was highly suspected • hVISA and VISA isolates less susceptible to many antibiotics, and are more likely to have SCCmec II or III element	Huang et al. (2016)
			MRSA isolates (bloodstream infection) (Brasil)	Agar screen E-test PAP	Genotypic	• Only 1(3%) of 31 isolates showed HR, demonstrating to be SCCmec II positive	Da Costa et al. (2016)
			Clinical isolate (Chile)	E-test	Unknown	• The hVISA isolate showed MIC above 8 mg/L for vancomycin and teicoplanin	(Vega et al. 2015)
			Clinical MRSA isolates (Asian countries)	E-test PAP	Unknown	• Sixteen (3.5%), among 462 isolates, showed HR	(Chung et al. 2015)
			hVISA isolates (varied sources) (Brasil)	Disc diffusion PAP	Genotypic	• Predominance of SCCmec type II, which is associated with hVISA and with higher mortality rates	Silveira et al. (2015)
		Teicoplanin	MRSA isolates (bacteraemia) (India)	E-test	Genotypic	• Mutations in the tcaA (transmembrane protein) and in the tcaB genes (multidrug efflux pump protein)	Bakthavatchalam et al. (2018)
	Lipopetides	Daptomycin	Clinical isolates (bacteraemia)	PAP	Genotypic	• Intrinsic HR could be induced after exposure to daptomycin • Mutations in *mgt, aspS and* prsA genes	Ji et al. (2020)
			Clinical isolates (bacteraemia) (China)	PAP	Unknown	• The heteroresistant subopulation greatly expanded in daptomycin agar containing 2–16 mg/L	Shafiq et al. (2017)
			MRSA isolates (blood) (Italy)	BMD E-test PAP	Phenotypic and Genotypic	• All isolates showed hVISA • Upregulation of genes involved in cell wall turnover and cell membrane perturbation (*rpoB* and *mprF*)	Capone et al. (2016)
			MRSA isolates (blood)	E-test PAP	Genotypic	• Previous exposure to teicoplanin may have selected for hVISA • Overexpression of *mprF and* dltA genes	Chen et al. (2015)

(*continued*)

Table 2. Continued.

Pathogen	Drug category	Antimicrobial agent	Sample source	Methods	HR mechanism	Observations	Ref.
	Lipopetides and Glycopeptides	Daptomycin Vancomycin	MRSA isolates (varied sources) (Brasil)	Agar screen BMD PAP	Phenotypic and Genotypic	• Increased expression of genes involved in cell wall metabolism, cell wall thickening, reduction of autolysis • SNPs in the rpoB and mprF genes *compared* with the susceptible strain	Okado et al. (2018)
	Folate pathway inhibitor	SXT	Clinical isolates (varied sources (Germany)	BMD Disc diffusion	Genotypic	• All 19 isolates showed HR and harboured one of the trimethoprim resistance *genes* dfrG *or* dfrS1 • Three isolates had an amino acid exchange in their FolP sequence, known to confer sulphonamide resistance	Scholtzek et al. (2020)
			Clinical isolates (nasal swabs) (African countries)	E-test PAP	Genotypic	• HR linked to the presence of dfrG (78%), dfrA (19%) or both (3%) resistance genes	Coelho et al. (2017)
	Tetracyclines	Omadacycline	MRSA and MSSA clinical isolates (China)	PAP	Genotypic	• Gene mutation in fibronectin-binding protein (FnBP) • Overexpression of genes encoding efflux pumps (branched-chain amino acid transport system II carrier protein and Na/Pi cotransporter family)	Bai et al. (2019)
		Eravacycline	Clinical isolates (varied sources) (USA)	Agar dilution PAP	Genotypic	• Transcriptional overexpression experiments indicated that USA300HOU_RS00550 (an Na/Pi cotransporter family protein) and USA300HOU_RS01625 (a branched-chain amino acid transport system II carrier protein) contributed to Erava HR	Wang et al. (2020)
			MRSA and MSSA clinical isolates (China)	Agar screen	Genotypic	• HR-derived clones had no 30S ribosome subunit mutations but their MICs were suppressed dramatically in the presence of efflux protein inhibitors	Zhang et al. (2018)

BMD: broth microdilution; HR: heteroresistance; hVISA: heteroresistant vancomycin-intermediate *S. aureus*; MDR: multidrug resistant; MRSA: methicillin-resistant *S. aureus*; PAP: population analysis profile; SCC*mec*: Staphylococcal cassette chromosome *mec* typing: SMX: sulfamethoxazole; SNPs: single nucleotide polymorphisms; SXT: trimethoprim/sulfamethoxazole; TZP: Piperacillin-tazobactam; VISA: Vancomycin-intermediate *S. aureus*.

phosphoethanolamine (pEtN), aiming at reinforcing the outer membrane, while neutralising the negatively charged lipid A and reducing colistin binding and cell leakage/death (Formosa et al. 2015; Kang et al. 2019).

Alongside to alterations in the lipid A moiety (Jayol et al. 2015; Cheong et al. 2019), other studies have reported another source of acquired colistin HR in *K. pneumoniae*, which occurs through the inactivation of MgrB. This transmembrane protein functions as a disruptor of the negative feedback loop of the PhoPQ two-component system (Halaby et al. 2016; Bardet et al. 2017). This causes the up-regulation of *pho*P-associated genes, creating more positively charged LPS and reducing its affinity to positively charged polymyxins (Baron et al. 2016; Jeannot et al. 2017).

In addition, colistin HR in *K. pneumoniae* has also been linked to mutations in *lpxM* and *yciM* (Halaby et al. 2016) and in CrrAB (Cheng et al. 2016; Cain et al. 2018). Intriguingly, other non-chromosomal determinants, such as capsule-hyperproduction or overexpression of RND-type efflux pumps have also been associated to the colistin-heteroresistant phenotype in *K. pneumoniae* (Poirel et al. 2017; Ernst et al. 2020). Colistin HR in *K. pneumoniae* can sometimes go unnoticed with resistant subpopulations requiring extended incubation times (48 h) to grow in broth dilution assays (sometimes showing incongruent results in methodologies using solid substrates) (Band et al. 2018). Moreover, the heteroresistant phenotype can be highly unstable in *K. pneumoniae* isolates, which is of great concern, as it causes unexplained *in vivo* treatment failure (Band and Weiss 2019).

Reported HR to other antimicrobials (e.g. tetracyclines) is shown to be mediated by either OqxAB and MacAB efflux pumps or by unstable tandem gene amplifications (Nicoloff et al. 2019).

Pseudomonas aeruginosa

P. aeruginosa stands out in a wide variety of life-threatening acute chronic infections in critically ill and immunocompromised patients (e.g. CF lung infection, otitis media, burn wounds, urinary tract and gastrointestinal infections) (Moradali et al. 2017; Azam and Khan 2019). The management of its infections has relied on several antibiotic categories, including aminoglycosides, cephalosporins, fluoroquinolones, penicillin with β-lactamase inhibitors (e.g. ticarcillin piperacillin in combination with clavulanic acid or tazobactam), monobactams, fosfomycin, and polymyxins (Bassetti et al. 2018). However, the extreme aptitude of *P. aeruginosa* to exhibit multiple intrinsically chromosome encoded mechanisms (e.g. efflux pumps, antibiotic-inactivating enzymes, target alterations, decreased permeability) and genetically imported elements (e.g. plasmids) carrying resistance has been jeopardising the effect of these drugs.

HR emergence in *P. aeruginosa* has become obvious as a consequence of its exposure to a high number of drugs. Analogously to *A. baumannii* and *K. pneumoniae*, colistin HR has been reported in this species. Accordingly, additional l-Ara4N moieties in lipid A profiles were demonstrated in *P. aeruginosa* heteroresistant isolates, corroborating the fact that mutations in both PmrAB and PhoPQ two-component regulatory systems are a common mechanism by which Gram-negative bacteria gain colistin resistance (Lin et al. 2019). Furthermore, two new regulatory systems (ParRS and CprRS), functioning as cationic peptide sensors, were found to also play a role in mediating HR in *P. aeruginosa* (Lin et al. 2019).

Besides colistin, HR to β-lactams has also been reported. Studies confirmed that AmpC and cephalosporinase overexpression or efflux system upregulation largely contributes to cefepime HR (Hocquet et al. 2006; Endimiani et al. 2008; Jia et al. 2020). Similarly, HR to carbapenems (as well as other antimicrobials) in *P. aeruginosa* involves remarkable levels of intrinsic Mex-Opr efflux pump systems (Mei et al. 2015; He et al. 2018; Qin et al. 2018) or truncation of OprD porin (Qin et al. 2018). Finally, mutations in the *gyrA* and *gyrB* target genes were found to play important roles in ciprofloxacin HR, as fluoroquinolones act by inhibiting the intracellular targets DNA gyrase and topoisomerase IV, inhibiting DNA replication (Feng et al. 2019).

Staphylococcus aureus

S. aureus is widely encountered in hospital and community settings, being closely associated with moderate to severe invasive infections (Liu et al. 2011). Methicillin-resistant *S. aureus* (MRSA) has growing as a leading cause of HAIs, being more likely to cause serious complications, such as blood sepsis, pneumonia, and endocarditis (Siddiqui and Koirala 2019).

Vancomycin is still the foremost therapeutic agent in serious MRSA infections, yet the ascending resistance towards this glycopeptide has made its use questionable. In fact, MRSA infections with reduced susceptibility to vancomycin have emerged in recent years (Table 2). Particularly, the emergence of the heteroresistant vancomycin intermediate *S. aureus* (hVISA), associated to prolonged bacteraemia, has contributed to higher rates of poor clinical outcomes

(Sakoulas et al. 2004; Maor et al. 2009; Claeys et al. 2016). The mechanism by which hVISA occurs is still unknown, partly explaining why the mechanistic basis of HR is often unstressed in many studies (Table 2). One proposed mechanism is the selective pressure from ever-present and longstanding vancomycin exposure (Khatib et al. 2015; Di Gregorio et al. 2016; Martirosov et al. 2017). Studies have also described various pathways involved in glycopeptides HR, namely cell wall thickening (Cui et al. 2003), slow growth rate (Basco et al. 2019), and altered peptidoglycan cross-linking (Di Gregorio et al. 2017). Genes related to bacterial cell regulation pathways (*vraSR, graSR, saeSR, agr*) (Bakthavatchalam et al. 2016, 2018; Di Gregorio et al. 2016; Kim et al. 2017), as well as the presence of the mobile genomic island staphylococcal cassette chromosome (SCC*mec*) types I and/or II, carrying genes related to methicillin (and other drugs) resistance (Silveira et al. 2015; Da Costa et al. 2016; Huang et al. 2016) have been documented.

HR to daptomycin, a cyclic lipopeptide used as alternative to treat MRSA infections, has been increasingly seen in MRSA isolates. This HR has been linked to changes in cell wall composition, charge, and fluidity (Capone et al. 2016; Okado et al. 2018), as well as to mutations in the *mprF* gene, resulting in increased lysyl-phosphatidyl glycerol production, and in the *rpoB/C* genes, encoding bacterial RNA polymerase subunits (Chen et al. 2015; Okado et al. 2018), in addition to a number of other gene mutations (Ji et al. 2020).

Recently, tetracycline HR has also arisen in both MRSA and methicillin-susceptible *S. aureus* (Zhang et al. 2018; Bai et al. 2019). The associated mechanisms are likely related to drug extrusion through bacterial efflux pumps (Zhang et al. 2018; Bai et al. 2019).

Candida species

HR has also been witnessed in eukaryotic pathogens, although at extremely rare frequencies. Ben-Ami et al. (2016) showed that fluconazole HR in *C. glabrata* was fundamentally genotypic, derived from high levels of efflux pumps, but also epigenetic. Azoles, such as fluconazole, inhibit the activity of the lanosterol 14-α-demethylase, enzyme responsible for ergosterol biosynthesis, leading to the accumulation of the toxic sterol 14-α-methyl-3,6-diol and loss of membrane integrity (Vieira and Nascimento 2017). The widespread and inadequate use of antifungals may explain the inefficacy of *Candida* infection treatments.

Antimicrobial specific analysis of HR

Over the last 5 years, HR to a panoply of antimicrobials has been reported (Figure 2). Glycopeptides rank first (31.4%) due to accumulating studies reporting hVISA. β-lactams stand second, with 18.6% of reported HR cases. Among them, carbapenems (e.g. imipenem, meropenem) team up with the highest proportion (15.4%), assuming particular concern in Gram-negative bacteria. Antimicrobial peptides assume an equal ratio than that found for carbapenems. Particularly, the dramatic escalation in HR cases towards the last-resource drug colistin has sounded the alarm to rethink new effective strategies against MDR strains. Finally, aminoglycoside HR has an estimated proportion of 6.4%, while other agents, including synthetic tetracyclines and sulphonamides, follow with an overall ratio of 6.9%. HR to quinolones (ciprofloxacin) (1.6%) and antifungals (fluconazole) (0.5%) is reported less.

It can be discerned from Figure 2 that nearly all the portraited categories of antimicrobials filling in the biggest "slices" of reported HR episodes are the ones interfering with bacterial membrane or cell wall, either by: i) binding to targets associated to cell membrane/cell wall function (e.g. glycopeptides; carbapenems); ii) acting as permeabilizers, displacing bacterial membrane architecture (e.g. colistin); or iii) interacting with the anionic cell surface whilst holding a very positive charge (cationic) at neutral pH, being known to be affected by membrane modification (aminoglycosides) (Krause et al. 2016). On the other hand, HR to agents targeting other vital cell components is less reported. Quinolones HR, for instance, derives from combined forms of resistance, namely target-mediated resistance (in gyrase and topoisomerase IV) along with cellular efflux pump overexpression and repression of porin channels (Aldred et al. 2014; Qin et al. 2018).

For a more complete overview, Table 3 summarizes HR incidences in clinically significant pathogens towards different antimicrobials, reported over the last 2 decades. There is an upward trend in the number of new reports, showing a growing interest for this topic, particularly with the advent of sophisticated diagnostic technologies. Reporting HR occurrences in pathogens with clinical importance and elucidating the underlying mechanisms would significantly enhance our understanding of HR implications in AMR development and its correlation with therapeutic failure, ultimately helping to guide effective therapeutic options.

Biofilm as a form of resistance

Biofilm development is a process whereby microorganisms irreversibly attach and grow on interfaces,

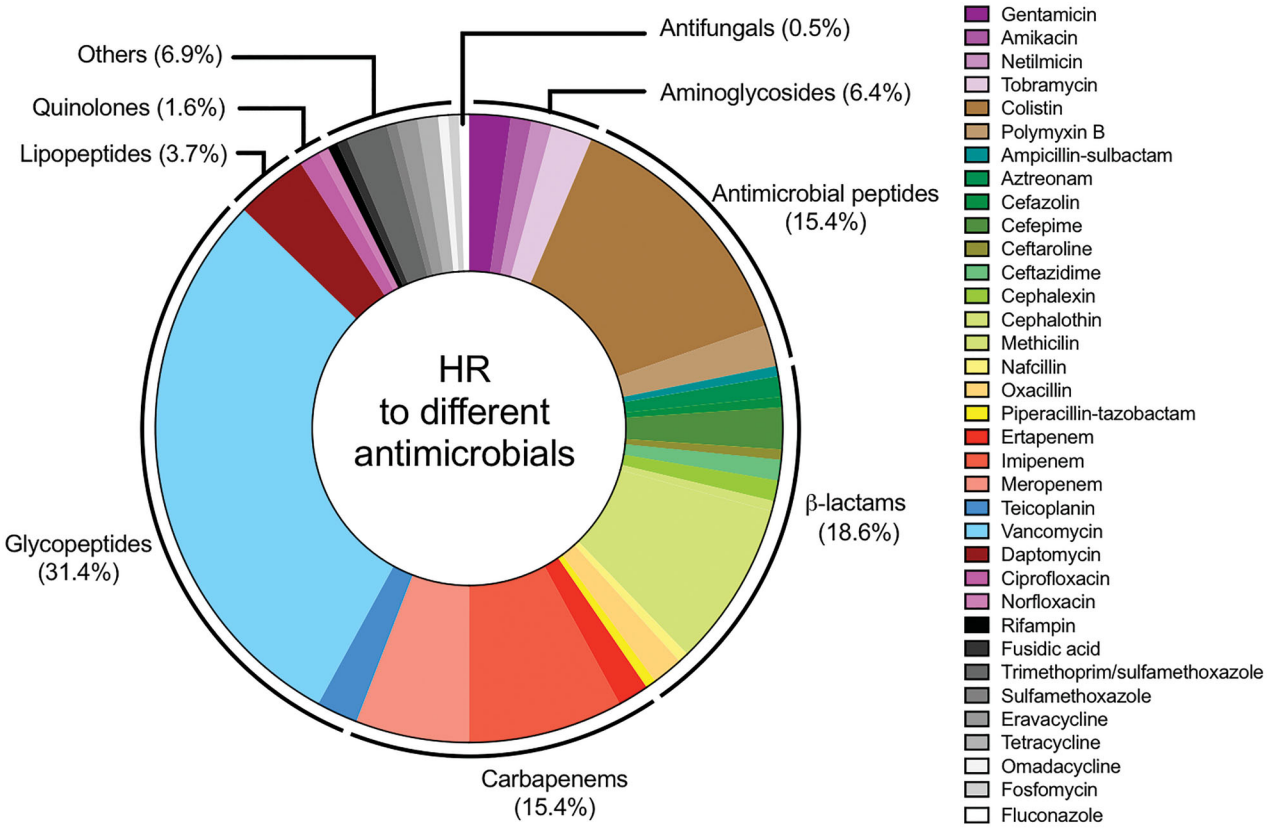

Figure 2. HR to different antimicrobials. Number of cases reporting antimicrobial HR, expressed as percentage, reported in clinically significant pathogens during the last 5-year period annotated in this review.

clumping as well-organized multicellular aggregates or layers with distinct architectures to persist in a wide range of environments (Lebeaux et al. 2014; Flemming and Wuertz 2019). These microorganisms are sheltered and spatially confined in a self-produced polymeric matrix, representing a major healthcare concern owing to their detrimental role in many infections, namely those associated with medical devices (Lebeaux et al. 2014). Pathogens grow in biofilms as a form of adaptive resistance and as a versatile strategy to invade the human body, becoming impervious to host defences and external stressors, such as antimicrobials (Bisht and Wakeman 2019). Biofilm-associated infections are not easily eradicated by conventional treatments, as microorganisms living as biofilms are 10- to 1000-fold more tolerant to antimicrobials than their free-living counterparts (Romling et al. 2012; Lopes et al. 2014).

A significant mechanism contributing to the increased AMR found in biofilms is the restricted drug penetration through the exopolysaccharide matrix. This matrix fulfils a variety of functions, ranging from structural robustness and protection from environmental stresses to gene regulation and nutrient adsorption (Hobley et al. 2015). Remarkably, the matrix is proficient in enmeshing microorganisms into a diffusion-limiting area, sparking microbial organization into a wide range of physiological states. Consequently, biofilms become inherently heterogeneous communities, even when formed by a single species (Stewart and Franklin 2008). Biofilm heterogeneity results from a highly complex interplay between stochastic stressors, such as spontaneous mutations, external stimuli factors (e.g. host immune system, antimicrobial treatment), internal microenvironment (oxygen, nutrient, and pH gradients), and interspecies interactions (Lebeaux et al. 2014; Bisht and Wakeman 2019). Taken together, these processes lead to the formation of widely distinct subpopulations within the biofilm strata with diversified metabolic profiles, genetic programs, spatial segregation, and differential stress responses (Hall-Stoodley et al. 2004; Pamp et al. 2008; Stewart and Franklin 2008; Høiby et al. 2010; Yan and Bassler 2019). The higher the biofilm biomass, the more evident this interplay and the larger the number of emerging subpopulations will be (Bisht and Wakeman 2019).

However, increased amount of biomass does not necessarily means increased AMR (Naparstek et al. 2014), but rather increased heterogeneity. Typically, a top-to-bottom gradient of decreasing susceptibilities can be found in most biofilms. The selective pressure

Table 3. Overview of reported antimicrobial HR incidences in clinically significant pathogens.

	Pathogen					
Drug category	*A. baumannii*	*Burkholderia* spp.	*Candida* spp.	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*
Aminoglycosides	Amikacin Gentamicin Netilmicin Tobramycin	Gentamicin	n/a	Amikacin Gentamicin Netilmicin Tobramycin	Tobramycin	n/a
Antimicrobial peptides	Colistin Polymyxin B	Polymyxin B	n/a	Colistin	Colistin Polymyxin B	n/a
β-lactams	Ampicillin- sulbactam Cefepime	Ceftazidime	n/a	Aztreonam	Aztreonam Cefepime Ceftazidime Piperacillin-tazobactam	Methicillin Cephalexin Oxacillin Cephalothin Nafcillin Cefazolin Ceftaroline
Carbapenems	Imipenem Meropenem	n/a	n/a	Ertapenem Imipenem Meropenem	Imipenem Meropenem	n/a
Glycopeptides	n/a	n/a	n/a	n/a	n/a	Vancomycin Teicoplanin
Lipopeptides	n/a	n/a	n/a	n/a	n/a	Daptomycin
Quinolones	n/a	Norfloxacin	n/a	n/a	Ciprofloxacin	n/a
Others	TMP-SMX	Rifampin	Fluconazole	Eravacycline Tetracycline SMX	Fosfomycin	Fusidic acid Omadacycline Eravacycline TMP-SMX

SMX: Sulfamethoxazole; TMP-SMX: Sulfamethoxazole/trimethoprim; n/a: not annotated.

provided by the accumulation of intercellular signalling molecules and waste products, along with oxygen and nutrient gradients, lead biofilm subpopulations to display differential growth and metabolic rates, ultimately antagonising the action of antimicrobials (Borriello et al. 2004; Stewart and Franklin 2008; Ryall et al. 2012; Jensen et al. 2017). In *P. aeruginosa* biofilms, for instance, metabolic active bacteria are mostly located in the outer layers, whereas low or non-metabolic active cells are in the lower regions (Xu et al. 1998; Walters et al. 2003; Bagge et al. 2004; Borriello et al. 2004; Williamson et al. 2012). In this biofilm arrangement, most antibiotics targeting biological processes during aerobic respiration (e.g. DNA replication, translation, cell wall synthesis) are only capable of clearing the outer layers (Brooun et al. 2000; Walters et al. 2003; Williamson et al. 2012; Soares et al. 2019). However, biofilm physiological stratification is not always consistent among species. In *Escherichia coli* macrocolonies, the active growing cells are located in the lower layers, while non-growing starving bacteria inhabit the upper ones in response to a diffusion-based nutrient supply (Serra et al. 2013). Other distinct biofilm stratifications include the three-layer patterns discerned in *S. aureus*, *S. epidermidis*, and in β-lactamase-negative *K. pneumoniae* microcolonies biofilms (Anderl et al. 2003; Rani et al. 2007). In the case of *S. aureus*, bacterial cells preferentially grow aerobically and fermentatively in layers near to air interfaces, whereas non-growing cells are sustained in the middle layers (Rani et al. 2007).

Heterogeneity in resistance levels within biofilms can be exhibited in a growth state-dependent manner. This form of resistance results from nutritional and oxygen constraints within the biofilm microenvironments or from antimicrobial exposure, which drive a small fraction of the population to enter a dormant or slow-growth state (persisters) (Allison et al. 2011; Conlon et al. 2015; Hall and Mah 2017; Soares et al. 2019). Once persisters are capable of surviving to lethal antimicrobial concentrations (Conlon et al. 2015; Soares et al. 2019), they are responsible for relapsing many biofilm-associated infections (Conlon 2014; Gollan et al. 2019). As such, the physiological heterogeneity in biofilms causes differential yet concerted resistance and tolerance mechanisms (Jorge et al. 2019). Regrettably, the contribution of biofilm subpopulations and their inherent variability to HR emergence is still poorly explored. While significant progress has been made in exploiting worldwide HR incidences, the fact is that the bulk of research is focused in planktonic microorganisms. So far, there is only one report addressing HR in biofilms (Silva et al. 2016), wherein heteroresistant subpopulations with ability to grow under colistin concentrations up to fourfold the MIC were found in *K. pneumoniae* biofilms. The resistant subpopulation exhibited a distinguishable colony morphology from the remaining biofilm population in the form of stable small-colony variants. These latest findings are reasonable grounds to anticipate occurrence of HR within biofilms, with the tremendous heterogeneous physiologies typically found in biofilms predicting the onset of such

phenotype. Taking this premise into consideration, we propose a theoretical model representing the heterogeneous physiologies that might arise in biofilms (Figure 3).

A total of five distinct AMR patterns can arise in polymicrobial biofilms, comprising: susceptible, resistant, and tolerant populations; a highly tolerant persister subpopulation; and a highly resistant subpopulation (HR subpopulation) (Figure 3(A)). When biofilms are exposed to antimicrobials, metabolically active susceptible cells, largely located at the top tiers, are promptly killed, whereas both resistant and heteroresistant cells are able to replicate under antimicrobial stress. Persisters, however, remain in a dormant state, not disturbed by the antimicrobials. Tolerant cells are able to survive antimicrobial action, however taking a longer time to grow (Figure 3(B)). When antimicrobial pressure drops, the resistant populations endure with their previous replicated numbers (in case of adaptive resistance, cells return to a susceptible profile). Usually, heteroresistant subpopulations, if unstable, revert to their original susceptibility patterns (Figure 3(C)). This selection for resistant, tolerant, and persister cells is a major concern in antibiotherapy and commonly the reason for infection recalcitrance and chronicity.

Polymicrobial biofilms in chronic infections

The notion that biofilms rarely exist as single-species entities and that most persistent infections are mediated by complex heterogeneous polymicrobial communities spanning different phylogenetic kingdoms is now widely recognized (Peters et al. 2012; Filkins and O'Toole 2015; O'Donnell et al. 2015; Kline and Lewis 2016). The presence of different microorganisms living in close proximity complicates the clinical picture of these polymicrobial infections (Lopes et al. 2012; Lopes, Azevedo, et al. 2017; Stacy et al. 2016). These type of infections have been reported to cause even worse outcomes than single-species infections (Pammi et al. 2014; Limoli et al. 2016; Limoli and Hoffman 2019) and are often associated to higher mortality rates. Microorganisms interact within the infection niche, augmenting the tenacity and recalcitrance of the infection (Azoulay et al. 2006; Hamet et al. 2012; Wolcott et al. 2013; Schroeder et al. 2017), paving the way for AMR evolution. These interactions can cause tremendous disturbance in microbial physiology in ways that affect disease progression outcome, by means of virulence factor upregulation (Duan et al. 2003; Sibley et al. 2008; Korgaonkar et al. 2013), altered biofilm formation (Ryan et al. 2008; Bragonzi et al. 2012; Lopes et al. 2012), and altered antibiotic susceptibility (Beaudoin et al. 2017; Lopes, Azevedo, et al. 2017, Lopes, Rodrigues, et al. 2017; Orazi and O'Toole 2017; Orazi et al. 2019).

Indeed, a striking characteristic of most chronic infections mediated by polymicrobial biofilms is their unsuccessful eradication by conventional therapy. The mechanisms that lead to unexplained treatment failures and poorer clinical outcome remain unclear, with HR as a likely cause. In addition to suboptimal therapeutic dosages that ignore the high resistance and tolerance levels of biofilms, there are other reasons for antibiotic treatment failure. They are related to the selection of the resistant cells within biofilms and the dissemination of resistance traits due to facilitated interactions among microorganisms (El-Halfawy and Valvano 2011, 2015). HR within a clonal cell population, despite rarely reported in biofilms, has been documented for several opportunistic pathogens with prominent roles in these infections, dramatically impacting the chronicity of such illnesses (Fusco et al. 2009; Higgins et al. 2010; Cheong et al. 2011; Sola et al. 2011; Campanile et al. 2012; El-Halfawy et al. 2013; Silveira et al. 2015; Band et al. 2016, 2018; Huang et al. 2016). Predictably, highly resistant cells that make up heteroresistant subpopulations may further complicate polymicrobial infections by affording protection to more susceptible cells (from the same or different species), either by counteracting antimicrobial effects through the release of neutralising elements or by means of metabolic (cooperative) interplay and cell–cell communication *via* sharing genetic material/resistance genes (Dufour and Rao 2011). This issue is discussed next.

Resistance spreading through interspecies interactions – clinical magnitude and implications in HR

The emergence of dissimilar levels of resistant phenotypes in heteroresistant populations has been assumed to be a reflection of variations in stochastic processes, in environmental metabolic clues, and of genetic architecture (circuits) (Norman et al. 2015; Dewachter et al. 2019). HR mechanisms, despite still unclear, presumably fall into 3 major categories: i) drug target modification; ii) drug inactivation; and iii) reduction of intracellular drug concentration (Band et al. 2016; Nicoloff et al. 2019). Also, HR (monoclonal) may have a phenotypic (i.e. non-genetic) or a genetic basis. Regardless of HR stability, it can, unfortunately, promote adaptive evolution. Under drug stress, the development of high rate cumulative mutations by resistant subpopulations may result in adaptive benefit to the whole population by

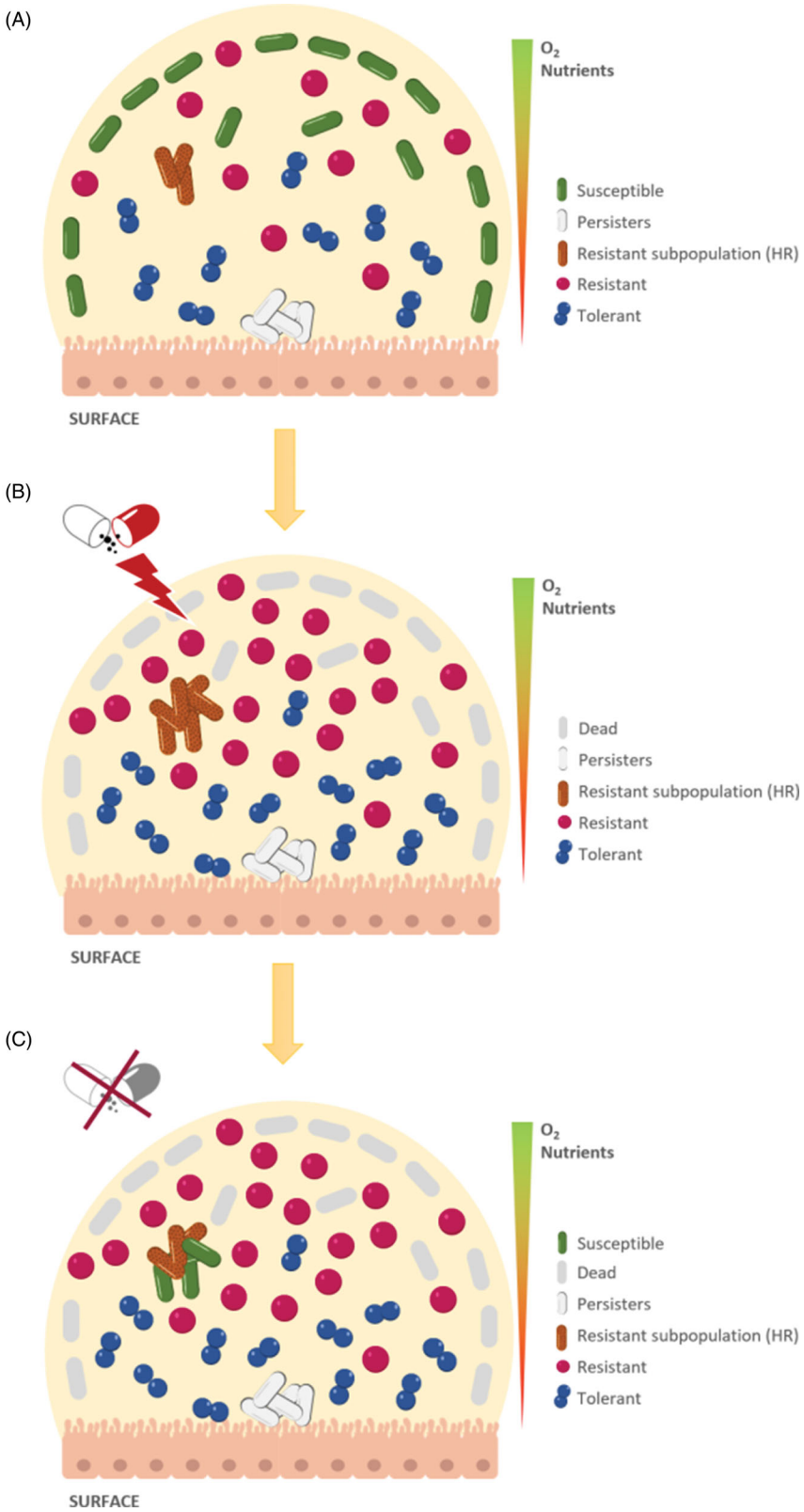

Figure 3. Heterogeneous physiologies in biofilms. (A) Five distinct patterns of AMR can arise simultaneously in biofilms when a theoretical minimum of three different species or strains are present: susceptible or metabolically active population (dark solid bacilus with shadowing); resistant (coccus) tolerant (diplococcus) populations; persister subpopulation, preferentially located at the bottom layers (light solid bacilus with shadowing); a heteroresistant (patterned bacilus with shadowing) subpopulation (HR). (B) Under antimicrobial selective pressure, metabolically active cells are killed, both resistant HR populations are able to replicate. Contrariwise, persisters are not affected by the antimicrobial activity tolerant cells are able to survive however exhibiting slow

Figure 3. (*Continued*)
growth. (C) When antimicrobial pressure drops, the resistant population maintains its numbers (however, some cells with adaptive resistance may revert to susceptible); HR subpopulation reverts to their original susceptibility patterns. Of note, it was intended to represent all conceivable AMR patterns that can be present at a biofilm. Bearing in mind that resistance tolerance are reflected in entire populations, we differentiate cell shapes into bacillus, coccus, diplococcus, to represent different species. Therefore, this model can be representative of a three-species biofilm. It is important to underscore that the top-to-bottom gradient antibiotic susceptibilities in this model may do not reflect what occurs in several biofilm mixed-species populations (in some cases, one species may overlay another).

augmenting its resistance or inducing HR in neighbouring cells (Li et al. 2017; Dewachter et al. 2019). Looking into co-infection scenarios, this review intends to pinpoint mechanisms of microbial resistance communication to gain insights into the implications that interspecies interactions may have on HR and on the outcome of polymicrobial infections.

Genetic mechanisms of resistance spreading

Even though HR stability is generally disregarded, several mechanisms have been proposed. Mutations on resistance genes, through either single-nucleotide polymorphisms (SNPs), frameshifts, or insertions/deletions, generate a stable HR, causing subpopulations to retain their resistance when antibiotic selective pressure drops. Unstable HR, on the contrary, is largely linked to tandem and unstable gene amplifications, although point mutations, insertion sequences, and small deletions can also occur. These genetic events explain why HR is highly unstable and transient in nature, frequently evading routine diagnostic tests (Andersson et al. 2019). In addition, gene modifications are generally intrinsic, conferring unfavourable effects by occurring at high rates through a disproportionate crossing over between homologous amplified gene copies (Adler et al. 2014; Reams and Roth 2015; Nicoloff et al. 2019). The resistant subpopulations with amplifications in regulatory genes (Landman et al. 2013; Jayol et al. 2015; Halaby et al. 2016), resistance genes encoding efflux systems (Chen et al. 2017; Zheng et al. 2018), and/or antibiotic/target-modifying or antibiotic-degrading enzymes (Sandegren and Andersson 2009), are selected and enriched under drug selective pressure. Whenever pressure drops, susceptible revertant cells are forced to reduce the number of gene copies and decelerate or suppress their growth (Sandegren and Andersson 2009; McGann et al. 2014; Nicoloff et al. 2019).

The fact that various genetic transfer mechanisms contribute extensively to resistance spreading in microbial populations is infamous. The frequency of inherited and spontaneous mutations and the horizontal exchange of resistance determinants is facilitated by high cell densities and their close proximity in polymicrobial biofilms (Hausner and Wuertz 1999; Driffield et al. 2008; Andersson and Hughes 2010; Orazi and O'Toole 2019). The emergence and dissemination of antibiotic resistance genes through horizontal gene transfer and integrative conjugative elements is well reported in these scenarios, converting biofilms into AMR hot spots (Li et al. 2018).

Considered a major pathway for spreading resistance genes, plasmid-mediated conjugation can transfer DNA between species from different genera and phyla (Sørensen et al. 2005). Biofilms are well suited for gene transferability, promoting abnormal rates of conjugation (Hausner and Wuertz 1999). One example comprises the transmission of an *E. coli* natural NDM-type plasmid encoding the carbapenemase-associated resistance gene $bla_{\text{NDM-1}}$ to *P. aeruginosa* and *A. baumannii* (Tanner et al. 2017). Another example is the acquisition of resistance towards vancomycin and other antibiotics, by *S. aureus*. This resistance is likely mediated through plasmid-horizontal transfer during the course of a polymicrobial biofilm infection. For example, the *vanA*-harbouring plasmid was presumably transferred from *Enterococcus faecium* to *S. aureus* (Weigel et al. 2007). Interestingly, the occurrence of *S. aureus* in heterogeneous biofilms tends to increase the rates of plasmid horizontal transfer, contributing to biofilm resistance (Venkatesan et al. 2015).

Usually, bacteria synthetize enzymes possessing deleterious effects against certain antibiotics. Within the various families of antibiotic-inactivating enzymes, β-lactamases are the most impacting ones. Particularly, the extended-spectrum β-lactamases (ESBL) are associated with major outbreaks of cephalosporin-resistant infections caused by ESBL-producing *E. coli* and *K. pneumoniae* (Sirot et al. 1988; Meyer et al. 1993). The genes encoding these enzymes were generally detected in plasmids that confer resistance to multiple antibiotic classes and are readily transferable among species (Sirot et al. 1987). β-lactamases have risen worldwide in pan-resistant Gram-negative pathogens, and their coding genes have spread into other *Enterobacteriaceae* as well as *P. aeruginosa* and *Acinetobacter* spp. (Queenan

and Bush 2007; Robledo et al. 2010). These findings strongly illustrate the potential for dissemination of resistance determinants between pathogens in the context of polymicrobial infections. Of note, a single conjugative event can have extensive implications in AMR spreading, not only within an infection but also between patients, having potential repercussions in antimicrobial treatment (Orazi and O'Toole 2019).

Non-genetic mechanisms of resistance spreading

Non-genetic mechanisms of resistance spreading are often dismissed, perhaps overshadowed by genetic machineries, making them not always clear. Nonetheless, there is increasing evidence showing modulation of population-wide resistance communication through non-genetic processes. Epigenetic inheritance is an example of non-genetic HR, and partially explains the vertical transmission of HR across generations without ensuring its stability (Dewachter et al. 2019). Another example involves the production and release of chromosomal- or plasmid-encoded enzymes (e.g. β-lactamases), particularly by Gram-negative bacteria, providing protection not only for the producer but also for neighbouring cells (Lee et al. 2013; Perez et al. 2014; Sorg et al. 2016; Kim et al. 2018). In biofilms, these enzymes assume particular importance as they accumulate within the matrix, degrading the antibiotic before it can reach the cells (Bush 2010; Høiby et al. 2010).

Microorganisms also possess signal transduction machineries called quorum-sensing (QS) that rely on chemical signals, enabling cell–cell communication and multicellular behaviour regulation. QS is required by prokaryotes and eukaryotes to coordinately respond to environmental stressors through the expression of genes mediating virulence and resistance traits (Abisado et al. 2018). Interspecies communication through QS signals impacts antibiotic sensitivity within polymicrobial biofilms, with one species offering protection to another. For example, *S. aureus* and *P. aeruginosa* develop intricate regulatory networks not only to evade and suppress each other (Hotterbeekx et al. 2017), but also to establish cooperative ecological relationships (Magalhães et al. 2019). HQNO (2-heptyl-4-hydroxyquinolone *N*-oxide), a molecule pertaining to the *Pseudomonas* quinolone signal (PQS) QS system pathway, shields *S. aureus* from multiple antibiotics (Hoffman et al. 2006; Orazi and O'Toole 2017). *P. aeruginosa* drives *S. aureus* towards fermentative metabolism and reduced ATP levels, resulting in staphylococcal reduced antibiotic sensitivity (Filkins et al. 2015; Radlinski et al. 2017). QS signals and related diffusible molecules can also impact antimicrobial efficacy by altering cell membranes. *S. maltophilia* provides *P. aeruginosa* resistance to polymyxins through signal factors that are perceived by *P. aeruginosa*, which responds by activating the two-component system PmrAB (Ryan et al. 2008).

Cross-kingdom interactions in polymicrobial infections may also enhance resistance and virulence mechanisms. For example, *C. albicans* protects *S. aureus* by synthetising the QS molecule farnesol, which decreases the susceptibility of *S. aureus* to vancomycin. Farnesol induces the production of reactive oxygen species in *S. aureus*, activating a general stress response, and upregulates bacterial efflux pumps (Kong et al. 2017). Another example is the dual-species biofilms encompassing *C. albicans* and *P. aeruginosa* that exhibits abnormal resistance towards antifungal–antibacterial (amphotericin B-polymyxin B) combinatorial therapy. Even though the mechanisms have not been fully elucidated, the authors proposed a mechanism whereby protection of one species to another confers augmented overall resistance (Lopes, Rodrigues, et al. 2017).

Several metabolites (e.g. indole, ammonia, nitric oxide, polyamines), secreted by bacteria or by host cells, act as cell signals and have been reported to influence biofilm formation and antimicrobial efficacy (Gusarov et al. 2009; Nijland and Burgess 2010; Lee and Lee 2010; El-Halfawy and Valvano 2012; Bernier and Surette 2013; El-Halfawy et al. 2013; Arora et al. 2015). For instance, indole, an aromatic compound produced by a vast number of bacteria (Lee and Lee 2010), can be sensed in a heterogeneous manner across different species. For example, indole is known to stimulate *P. aeruginosa* biofilm formation and, despite repressing QS-regulated virulence factors (e.g. pyocyanin, rhamnolipid, PQS, pyoverdine), to induce resistance to tetracycline, gentamicin, and ampicillin (Lee et al. 2009). Indole also protects persisters against oxidative stress (Vega et al. 2012).

Volatile compounds, such as ammonia, 2,3-butanedione, and polyamines, also constitute a large class of potential infochemicals with ability to shift antimicrobial susceptibility profiles within microbial communities (Kai et al. 2009; El-Halfawy et al. 2013). Volatile-mediated communication is particularly troublesome in that no intimate cell–cell contact is required. Exposure to biogenic ammonia, for instance, resulted in increased resistance to tetracycline in various Gram-negative and Gram-positive bacteria, including *P. aeruginosa* and *S. aureus* (Bernier et al. 2011). Polyamines, synthesized by

nearly all bacteria (Tabor and Tabor 1984), play important functions by regulating bacterial growth, biofilm formation, biosynthesis of siderophores, cellular differentiation, and AMR (Wortham et al. 2007; Kohanski et al. 2010; Johnson et al. 2012; Tkachenko et al. 2012). Given their ubiquitous occurrence in bacteria, their accumulation in the environmental milieu is anticipated. At infection sites, polyamines tissue levels increase significantly, contributing to interspecies communication in multiple ways (Zhang et al. 2000; Bjelaković et al. 2010). A great example demonstrating polyamines as great HR and resistance-spreading mediators across distinct bacterial populations was observed with putrescein. Secreted at abundant levels by a highly resistant subpopulation of *Burkholderia cenocepacia*, this polyamine provided antimicrobial protection to sensitive *P. aeruginosa*. This interaction relied on the synthesis of putrescein by the resistant subpopulation, acting in concert with the *Burkholderia* conserved protein YceI, to afford protection to the outer membrane of *P. aeruginosa* from polymyxin B attack (El-Halfawy et al. 2013).

Overall, these mechanisms partly account for the increased recalcitrance observed in polymicrobial biofilm-mediated infections. Furthermore, their disclosure may help decipher the role of these consortia as a "gold" opportunity for interspecies commutation of resistance determinants in heteroresistant populations, thus elevating its magnitude in endangering antimicrobial therapies of these infections.

Concluding remarks

It has been widely recognized that biofilms display high levels of heterogeneity at infection sites, consequently generating subsets of phenotypes specialized at surviving antimicrobial attacks. Here, we focussed on HR, a yet poorly understood phenomenon, in which only a subset of cells in a population is resistant to antimicrobial treatment. The continuous HR emergence in clinically relevant pathogens is alarming and might be a significant cause of therapeutic failure in chronic and recurrent infections, which are often polymicrobial. HR incidence in biofilms is still largely underestimated. Moving forward, we argue that this phenomenon should no longer be ignored, being of utmost importance that we thoroughly investigate all aspects of HR. To increase the chance of detecting HR, we suggest revising the criteria for HR identification in consortia, either through using clinically meaningful biofilm susceptibility endpoint parameters (e.g. extending cut-off values to minimum biofilm inhibitory concentrations rather than MIC), extending the biofilm incubation time (as HR has a prolonged lag phase comparatively to native populations), or both. To accomplish this, more accurate and sensitive diagnostic tools are needed for major advances. Only after having a comprehensible understanding of the molecular mechanisms underpinning HR, we will be allowed to discern how we might overcome them. Future research is also required to elucidate the molecular basis of HR communication and its role in resistance spreading. Only then we will be able to find strategies to counteract HR, through effective inhibitors of key cooperation pathways. This will hopefully also translate into a more uniform response to biofilm infection treatment and a reduction in therapeutic failure. At this moment, HR is still an emerging field in need of clarity.

Disclosure statement

The authors declare no potential conflict of interest.

Funding

This work was supported by the Portuguese Foundation for Science Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2020 unit BioTecNorte operation [NORTE-01-0145-FEDER-000004] funded by the European Regional Development Fund under the scope of Norte2020–Programa Operacional Regional do Norte. The authors also acknowledge COMPETE2020 FCT for the project POCI-01-0145-FEDER-029,841 and for the Scientific Employment Stimulus 2017 grant [CEECIND/01507/2017] (A. M. Sousa).

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